THE
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JOURNAL

Thermal proteome profiling of breast cancer cells reveals proteasomal activation by CDK4/6 inhibitor palbociclib

Teemu P. Miettinen, Julien Peltier, Anetta Härtlova, Marek Gierliński, Valerie M. Jansen, Matthias Trost and Mikael Björklund

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 30th October 2017

Thank you for the submission of your manuscript (EMBOJ-2017-98359) to The EMBO Journal. Your study has been sent to three referees, and we have received reports from all of them, which I copy below.

As you will see, the referees acknowledge the potential high interest and novelty of your work, although they also express a number of concerns that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In particular, referee #2 points out the need for you to corroborate the senescence phenotype observed and consolidate your findings on implication of ECM29 by gain-of-function analyses. Referee #1 is concerned about the CD4/6-dependence of palbociclib's effects and asks you to investigate further this aspect by additional experiments. In addition, all referees list a number of technical issues on assays used and controls made, that need to be addressed to achieve the level of robustness needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and we are the in principle happy to invite you to revise your manuscript experimentally to address the referees' comments.

REFEREE REPORTS

Referee #1:

The manuscript by Miettinen et al applies the recently developed Thermal Proteome Profiling (TPP) technology to elucidate the mode of action of the CDK inhibitor palbociclib. The authors find that cognate targets of palbociclib are affected in their thermal stability; additionally they observe stabilization of a number of other kinases. Intriguingly, they also find that the 20S proteasome complex changes its stability. The finding is followed and the following points are convincingly demonstrated:
1) Palbociclib treatment leads to activation the proteasome
2) The activation is not a consequence of inhibition of the cognate CDK targets.

3) The activation is due to dissociation of the proteasome with ECM29
4) ECM29 RNA levels turn out to some extent to be predictive of relapse free survival of endocrine treated breast cancer patients.
I am convinced by the biological findings and validations, and I find the fact that TPP can discern proteasome activity an exciting development of the technology. It still remains to be understood exactly what is the molecular mechanism of proteasome activation by palbociclib, but in my opinion it is too much to ask for this particular manuscript.
I recommend publication following some minor, but important changes to the manuscript:

From the manuscript: "When combined with mass spectrometry, the thermal stability changes in proteins can be quantified using a quantitative proteomic approach, holding great promise for 110 identifying drug targets in live cells (Franken, Mathieson et al., 2015, Savitski et al., 2014)."
Please be more complete in citations and also cite Becher et al Nat Chem Bio 2016 as this is a recent example of successful in situ off target identification and validation.

From the manuscript:
"Heat aggregated proteins were
135 removed by centrifugation and soluble proteins were analyzed by multiplexed quantitative mass spectrometry using isotopically labelled tandem mass tags."
Please add relevant references for TMT labeling

From the manuscript:
"When equal amount of protein is analyzed by mass spectrometry for each temperature point, thermally stable proteins display increased abundance with temperature in the raw data (Fig. 1a, middle)."
This is an interesting approach for normalization. One point I have is that at 65C you have very little protein left in the soluble fraction. If you want to take the same protein amount there as for 37C, you need much more starting material (number of cells), could you please comment on that?

From the manuscript:
"The remaining proteins with poorer quality thermal denaturation curves were enriched for membrane proteins and mitochondrial proteins as shown before (Savitski et al., 2014)"
This issue has since been resolved by using the mild detergent NP40 instead of only PBS for cell lysate. This has no impact on the conclusion drawn in this study. But I would still ask the authors to clarify that. The way the above sentence reads suggests that currently it is not possible to properly assess membrane proteins, which is not true.

Referee #2:

The paper (EMBOJ-2017-98359) entitled "Thermal proteome profiling of breast cancer cells reveals proteasomal activation by CDK4/6 inhibitor palbociclib" uses thermal profiling as a screening technique to better understand the molecular mechanism of the chemotherapeutic drug palbociclib. This is an important question because a more complete characterization of drug action can be used to identify patients most likely to benefit from its use. Overall this is an impressive paper. The amount of experimental data is extensive and of high quality, with well controlled experiments presented in a logical fashion. The results support the contention that a major unanticipated effect of palbociclib is proteasome activation, and further provides a mechanistic explanation for this phenomenon. A testament to its persuasion is that one is left wondering whether palbociclib designation as a CDK4/6 inhibitor is at all relevant to its clinical utility. The conclusions are significant because they can immediately impact which patient cohorts receive palbociclib, with the potential to enhance its efficacy and extend patient lifespan. In my opinion the manuscript is immediately acceptable for publication with no major changes. Minor comments and interesting questions (which do not need to be answered for publication) are listed below.

I am not by any means an expert on thermal profiling, and the following probably reflects my lack of understanding. However, it seems remarkable that this approach worked, given that it evaluates ligand induced protein thermal stability (105-107). While this can be indirect, in this case the effect (unidentified) results in dissociation of ECM29, which in turn results in thermal shifting of the 20S (not 19S) proteasome subunit (197-199). One can't argue too much with results, but it might be worthwhile to hypothesize why the 20S thermal stability should be affected.
264-266: The lack of increase in protein ubiquitination is curious, given that it is (generally, but not always) a pre-requisite to degradation. So, how can protein degradation increase if ubiquitinated proteins are not being generated at a higher rate? This scenario implies that proteolysis per se is rate limiting, and further that ubiquitinated proteins are lying around waiting to be degraded. Most work in the field suggests otherwise.
There are two general ways to interpret increased proteasome activity against artificial substrates. 1) More proteasomes are being inactivated from an inactive state (such that the total number of active proteasomes increases; or 2) Enhancing the catalytic rate of individual proteasomes (the number of total active proteasomes does not increase). Which explanation do the authors prefer?
400: Is ECM29 upregulated or showing enhanced association with the proteasome in the resistant cell lines?
Given the multiple effects of palbociclib and questions about the significance of CDK4/6 inhibition, it might be worthwhile to determine if palbociclib still has the observed effect on the proteasome in the absence of CDK4/6 (fig 4). In other words, perhaps the effect of palbociclib on CDK4/6 is not what we think it is (in cells).

Minor corrections/comments
31: a phenotype that are is not....
351: lower expression of ECM29 was associated
402: and, at least in MCF7, cells,.....
440: Nonetheless, the palbociclib-induced effect? Augmented proteolysis...
458: In contrast to recent work...This is not really a contrast, and the respective observations are compatible given the complex role of the proteasome in cell cycle progression.

Referee #3:

1-
General summary and opinion about the principle significance of the study, its questions and findings
In this manuscript, Miettinen et al. use a thermal proteome profiling approach to identify molecular mechanism(s) responsible for palbociclib-induced senescence in ER-positive breast cancer cells. Interestingly authors found that CDK4/6 inhibition activates the proteasome by an indirect mechanism that is mediated by the decreased abundance of the proteasomal inhibitor ECM29, ultimately inducing senescence.
The study is conceptually relevant, given the important that CDK4/6 currently have in the clinic and the need of better understanding the anti-cancer mechanisms these drugs. In general the conclusions are supported by the results. However the work raise some questions that should be addressed prior its publication in The EMBO Journal.

2-
specific major concerns essential to be addressed to support the conclusions
Despite being an interesting discovery, particularly given its clinical relevance, the implication of ECM29 in the senescence induction lack some depth. The reduced proliferation and enlarged cell morphology observed upon ECM29 loss indeed suggests features of senescence (Line 337 & Fig. 5d). However, when used as a single method, acidic beta-galactosidase activity might not be a reliable senescent marker, as it may simply indicate general cellular stress. In order to draw solid conclusions, authors should confirm the senescent state secondary to ECM29 loss with additional established senescence molecular markers (e.g., reduced RB phosphorylation, induction of p16, p21, Lamin B1 suppression, upregulation of SASP members).

The suggestion that patients with high levels of ECM29 would benefit the most for palbociclib treatment should be strengthened with gain of function experiments and possibly validated in vivo.

The methods section lacks detail on how the CRISPR edited MCF7 ECM29+/- cells were generated, if by stable integration or by transient transfection of the vectors into the cells. The appropriate control (particularly in the case of a stable integration) in Fig. 5c would be an MCF7 cell line edited with CRISPR/Cas9 for a non-targeted sequence delivered in the exact same way as for the ECM29+/- cells.
Authors did confirm the reduced proliferation phenotype of the ECM29 loss with siRNA using the appropriate siRNA control in Supplementary Fig. 7b. However, the level of ECM29 knockdown in these experiments is missing.

Given that the authors were not able to generate ECM29 homozygous knockout cells (most likely due to the lethal effects), did authors verified that the observed reduced proliferation illustrated in Fig. 5c and Supplementary Fig. 7b is not due to cell death/apoptosis of the gRNA-ECM29 or siRNA-ECM29 cells?

What was the cut off used to define high versus low ECM29 mRNA expression (Fig. 5e-g)?

What was the methodology used in the cell cycle analysis illustrated in Fig. 6c-e?

3-
minor concerns that should be addressed
Line 31 - "is" instead of "are"

Line 331 - Although the levels of ECM29 are about 50% reduced comparing to the WT cells (Western blot in Fig. 5c), it is not clear to the reader how did the authors conclude that the MCF7 CRISPR edited cells are heterozygous for ECM29 without a proper sequencing experiment to prove that.

Line 1055 - "levels from Western blots in (g)" instead of "(e)"

Response to reviewers regarding the manuscript by Miettinen, Peltier, et al.

The comments made by reviewers are in black and our responses are in red. Thank you for taking the time to review our work and prove constructive feedback. In the manuscript all changes have been highlighted with yellow colour.

Referee #1:

The manuscript by Miettinen et al applies the recently developed Thermal Proteome Profiling (TPP) technology to elucidate the mode of action of the CDK inhibitor palbociclib. The authors find that cognate targets of palbociclib are affected in their thermal stability, additionally they observe stabilization of a number of other kinases. Intriguingly, they also find that the 20S proteasome complex changes its stability. The finding is followed and the following points are convincingly demonstrated:
1) Palbociclib treatment leads to activation the proteasome
2) The activation is not a consequence of inhibition of the cognate CDK targets.
3) The activation is due to dissociation of the proteasome with ECM29
4) ECM29 RNA levels turn out to some extent to be predictive of relapse free survival of endocrine treated breast cancer patients.
I am convinced by the biological findings and validations, and I find the fact that TPP can discern proteasome activity an exciting development of the technology. It still remains to be understood exactly what is the molecular mechanism of proteasome activation by palbociclib, but in my opinion it is too much to ask for this particular manuscript.

We are grateful for the positive comments and, as shown below, we have addressed all the points raised by the reviewer.

I recommend publication following some minor, but important changes to the manuscript:

From the manuscript:
"When combined with mass spectrometry, the thermal stability changes in proteins can be quantified using a quantitative proteomic approach, holding great promise for identifying drug targets in live cells (Franken, Mathieson et al., 2015, Savitski et al., 2014)."
Please be more complete in citations and also cite Becher et al Nat Chem Bio 2016 as this is a recent example of successful in situ off target identification and validation.

We have now cited this paper as suggested.

From the manuscript:
"Heat aggregated proteins were removed by centrifugation and soluble proteins were analyzed by multiplexed quantitative mass spectrometry using isotopically labelled tandem mass tags."
Please add relevant references for TMT labelling

We now refer in this context the original paper by (Savitski et al., 2014), where the same TMT labelling approach was used as requested.

From the manuscript:
"When equal amount of protein is analyzed by mass spectrometry for each temperature point, thermally stable proteins display increased abundance with temperature in the raw data (Fig. 1a, middle)."

This is an interesting approach for normalization. One point I have is that at 65C you have very little protein left in the soluble fraction. If you want to take the same protein amount there as for 37C, you need much more starting material (number of cells), could you please comment on that?

We agree that at the highest temperatures, relatively little protein is left soluble. While this could potentially cause sensitivity issues for the mass spec analysis, it should have minor effect on the data normalization and fitting. We wanted to keep it simple and consistent by analysing same amount of starting material.

From the manuscript:
"The remaining proteins with poorer quality thermal denaturation curves were enriched for membrane proteins and mitochondrial proteins as shown before (Savitski et al., 2014)"
This issue has since been resolved by using the mild detergent NP40 instead of only PBS for cell lysate. This has no impact on the conclusion drawn in this study. But I would still ask the authors to clarify that. The way the above sentence reads suggest that currently it is not possible to properly assess membrane proteins, which is not true.

The reviewer is correct and we have now acknowledged this in the introduction by stating that "Furthermore, alternative sample preparation with NP40 has allowed improved detection of membrane proteins (Reinhard et al., 2015)."

Referee #2:

The paper (EMBOJ-2017-98359) entitled "Thermal proteome profiling of breast cancer cells reveals proteasomal activation by CDK4/6 inhibitor palbociclib" uses thermal profiling as a screening technique to better understand the molecular mechanism of the chemotherapeutic drug palbociclib. This is an important question because a more complete characterization of drug action can be used to identify patients most likely to benefit from its use. Overall this is an impressive paper. The amount of experimental data is extensive and of high quality, with well controlled experiments presented in a logical fashion. The results support the contention that a major unanticipated effect of palbociclib is proteasome activation, and further provides a mechanistic explanation for this phenomenon. A testament to its persuasion is that one is left wondering whether palbociclib designation as a CDK4/6 inhibitor is at all relevant to its clinical utility. The conclusions are significant because they can immediately impact which patient cohorts receive palbociclib, with the potential to enhance its efficacy and extend patient lifespan. In my opinion the manuscript is immediately acceptable for publication with no major changes. Minor comments and interesting questions (which do not need to be answered for publication) are listed below.

We thank the reviewer for the very positive comments. Below we have tried to address all the minor comments raised by the reviewer.

I am not by any means an expert on thermal profiling, and the following probably reflects my lack of understanding. However, it seems remarkable that this approach worked, given that it evaluates ligand induced protein thermal stability (105-107). While this can be indirect, in this case the effect (unidentified) results in dissociation of ECM29, which in turn results in thermal shifting of the 20S (not 19S) proteasome subunit (197-199). One can't argue too much with results, but it might be worthwhile to hypothesize why the 20S thermal stability should be affected.

In the absence of structural information, we can only speculate how ECM29 affects the thermal stability of the 20S subunit. Nevertheless, this finding is consistent with prior work showing that ECM29 interaction involves change in proteasome conformation (De La Mota-Peynado, JBC, 2013). We have now added this second point to the manuscript by stating that "ECM29 binding to the 20S proteasome also changes the proteasome conformation (De La Mota-Peynado, Lee et al., 2013), which likely explains the increased 20S thermal stability upon ECM29 dissociation from the proteasome." A potential way to address this would be through cryo-electron microscopy, but this would be a rather time consuming and laborious follow-up paper by itself based on discussion with an expert in the field.

264-266: The lack of increase in protein ubiquitination is curious, given that it is (generally, but not always) a pre-requisite to degradation. So, how can protein degradation increase if ubiquitinated proteins are not being generated at a higher rate? This scenario implies that proteolysis per se is rate limiting, and further that ubiquitinated proteins are lying around waiting to be degraded. Most work in the field suggests otherwise.

The reviewer is correct that this is an unexpected result. We do not want to claim that for all proteins the rate-limiting step in degradation is proteasome activity. However, substrate unfolding and entry into proteasome particle are also important for proteolysis (Dorn IT, et al., J Mol Biol, 1999. & Henderson A, et al., JBC, 2011). Considering that the presence of EMC29 on the proteasome is believed to close the substrate entry channel of the proteasome (De La Mota-Peynado, Lee et al., 2013), this data suggests that the entry into proteasome can limit the degradation of at least some proteins in a manner that is not inconsistent with the current understanding of ubiquitination being generally rate-limiting. Note that in order to explain our data, not all proteins have to be degraded faster when proteasome is activated. As palbociclib also differentially affects ubiquitin linkages, it is possible that this affects substrate degradation as, for example, K48/K63 branched linkages preferentially associate with proteasomes in cells (Ohtake F, et al., PNAS, 2018).

As this is an important point, we have now tried to clarify our manuscript by adding the following comments to the manuscript:

"As the rate of degradation for most proteins is regulated by ubiquitylation, not proteasome activity, the palbociclib induced proteasomal activation may only affect a specific subset of proteins, such as those with K48/K63 branched linkages (Ohtake, Tsuchiya et al., 2018)."

"Furthermore, our observation that palbociclib increases proteasome activity, but not ubiquitination levels, is consistent with the previous work suggesting that the presence of ECM29 on the proteasome induces a closed conformation of the substrate entry channel of the core particle (De La Mota-Peynado et al., 2013)."

There are two general ways to interpret increased proteasome activity against artificial substrates. 1) More proteasomes are being inactivated from an inactive state (such that the total number of active proteasomes increases; or 2) Enhancing the catalytic rate of individual proteasomes (the number of total active proteasomes does not increase). Which explanation do the authors prefer?

Our data does not directly provide any evidence to separate these two options and both of them might be true. The current literature on ECM29 suggests that at least option #2, where catalytic rate is increased, is likely true (ECM29 inhibits proteasomal degradation rate). At the same time, it may be that not all proteasomes are inhibited by ECM29 under normal conditions. The stoichiometry between ECM29 and proteasomes is not known, nor do we know if there are separate pools of

proteasome with and without ECM29. Thus, palbociclib or ECM29 knockdown may also change the relative abundances of proteasome pools with and without ECM29. While we agree that this is a very thought provoking question, it goes way beyond the scope of this manuscript.

400: Is ECM29 upregulated or showing enhanced association with the proteasome in the resistant cell lines?

Unfortunately, we do not have RNA-seq or MS data on these cells to fully answer this question. However, the resistant cell lines display overactive AKT pathway (Jansen, et al., 2017), which may explain the lower baseline proteasome activity through its effect on mTOR. If this is the case, the mechanism affecting the proteasome is distinct from palbociclib, as our data clearly shows that mTOR inhibition does not induce similar effects on ECM29 as palbociclib. Thus, we think it is unlikely that ECM29 is radically affected in the resistant cells, but rather the resistance is due to the altered baseline proteasomal (or ubiquitin pathway) activity.

Given the multiple effects of palbociclib and questions about the significance of CDK4/6 inhibition, it might be worthwhile to determine if palbociclib still has the observed effect on the proteasome in the absence of CDK4/6 (fig 4). In other words, perhaps the effect of palbociclib on CDK4/6 is not what we think it is (in cells).

This is a good point, which we have considered. As shown in figure 4, we have done this using knockdown experiments, which are not the perfect validation as some CDK activity might remain. However, knockout of both CDK4 and CDK6 completely blocks proliferation in breast cancer cells. This is the very rationale for the development of these Cdk4/6 inhibitors! This makes the use of CRISPR-Cas9 system a non-viable option, as the resulting population cannot be expanded sufficiently to carry out the experiments. We have also considered obtaining CDK4 and CDK6 null MEFs but, unfortunately, the one laboratory currently in the possession of those MEFs has not replied to our emails. While genetic models of Cdk4/6 inhibition clearly indicate that breast cancer cell proliferation is dependent on these Cdks, I think the big issue with palbociclib is that it is not such a specific inhibitor as previous literature made everyone to believe. Palbociclib's effects on proteasome and other kinases (as also demonstrated by a recent Science paper (Klaeger S, The target landscape of clinical kinase drugs. December 2017) showing that palbociclib was one of the most promiscuous kinase inhibitors) clearly demonstrates this. It is likely because of the lack of CDK4/6 specificity that it works so well in the clinic

Minor corrections/comments
31: a phenotype that are is not....
351: lower expression of ECM29 was associated
402: and, at least in MCF7, cells,.....
440: Nonetheless, the palbociclib-induced effect? augmented proteolysis...
458: In contrast to recent work...This is not really a contrast, and the respective observations are compatible given the complex role of the proteasome in cell cycle progression.

Thank you for pointing these minor corrections out to us.

Referee #3:

1-
general summary and opinion about the principle significance of the study, its questions and

findings
In this manuscript, Miettinen et al. use a thermal proteome profiling approach to identify molecular mechanism(s) responsible for palbociclib-induced senescence in ER-positive breast cancer cells. Interestingly authors found that CDK4/6 inhibition activates the proteasome by an indirect mechanism that is mediated by the decreased abundance of the proteasomal inhibitor ECM29, ultimately inducing senescence.
The study is conceptually relevant, given the important that CDK4/6 currently have in the clinic and the need of better understanding the anti-cancer mechanisms these drugs. In general the conclusions are supported by the results. However the work raise some questions that should be addressed prior its publication in The EMBO Journal.

We would like to thank the reviewer for positively acknowledging that our work is relevant and conclusion are supported by the data. Below we have addressed the points raised by the reviewer.

2-
specific major concerns essential to be addressed to support the conclusions
Despite being an interesting discovery, particularly given its clinical relevance, the implication of ECM29 in the senescence induction lack some depth. The reduced proliferation and enlarged cell morphology observed upon ECM29 loss indeed suggests features of senescence (Line 337 & Fig. 5d). However, when used as a single method, acidic beta-galactosidase activity might not be a reliable senescent marker, as it may simply indicate general cellular stress. In order to draw solid conclusions, authors should confirm the senescent state secondary to ECM29 loss with additional established senescence molecular markers (e.g., reduced RB phosphorylation, induction of p16, p21, Lamin B1 suppression, upregulation of SASP members).

We agree with the reviewer and to better address the role of ECM29 in senescence we have repeated our ECM29 knockdowns in MCF7 and performed additional senescence marker assays. In short, we examined two independent senescence markers, p21 protein levels and S139 phosphorylation levels of H2AX. Both of these markers were significantly upregulated as a consequence of ECM29 knockdown. These data have been added to Figure 5 (new panels e-g) and the following text has been added to the manuscript: “We further validated this by examining two other senescence associated markers, S139 phosphorylation of Histone H2A.X (γH2AX) and p21 protein levels (Lawless, Wang et al., 2010), in MCF7 cells where ECM29 was silenced using with siRNAs (Fig. 5e). Both of these markers displayed marked increase after silencing of EMC29 (Fig. 5f and Fig 5g) supporting our other results.”

The suggestion that patients with high levels of ECM29 would benefit the most for palbociclib treatment should be strengthened with gain of function experiments and possibly validated in vivo.

Again, we agree with the reviewer that gain-of-function experiments would strengthen our conclusions and we have carefully considered such experiment. However, we have not been able to obtain a gain-of-function model. This is likely due to two facts. First, ECM29 is a huge protein, approximately 205 kDa. This makes transfections and controlling the expression level of the protein ineffective. Second, even if proper expression levels were achieved, the ECM29 association with the proteasome may not increase which would be the pre-requisite for a gain-of-function phenotype. The mechanism which controls ECM29 association with the proteasome is not known and we thus do not have the required knowledge to create a gain-of-function system. We further believe that - as much as we would like these experiments - *in vivo* data would be out of the scope of this paper.

The methods section lacks detail on how the CRISPR edited MCF7 ECM29+/- cells were generated, if by stable integration or by transient transfection of the vectors into the cells. The appropriate control (particularly in the case of a stable integration) in Fig. 5c would be an MCF7 cell line edited with CRISPR/Cas9 for a non-targeted sequence delivered in the exact same way as for the ECM29+/- cells.

Thank you for pointing this out. We generated these cell lines using transient transfection and thus we believe that the WT cells without transfection of Cas9 and a non-targeted sequence represents a more accurate control. We have now added further details of the CRISPR cells in the methods section. We generated two independent cell lines both displaying approximately 50% reduction and show data with only one of these as they performed identically in preliminary tests.

Authors did confirm the reduced proliferation phenotype of the ECM29 loss with siRNA using the appropriate siRNA control in Supplementary Fig. 7b. However, the level of ECM29 knockdown in these experiments is missing.

We have now added western blots displaying the knockdown efficiency after 48h RNAi. These siRNA mediated knockdown worked robustly in our hands and we did not therefore see the need to quantify the knockdown levels each day of the experiment.

Given that the authors were not able to generate ECM29 homozygous knockout cells (most likely due to the lethal effects), did authors verified that the observed reduced proliferation illustrated in Fig. 5c and Supplementary Fig. 7b is not due to cell death/apoptosis of the gRNA-ECM29 or siRNA-ECM29 cells?

The RNAi, knock-out and drug experiments indicate that the cells do not proliferate as they exit the cell cycle and become quiescent rather than die. When analysing gRNA-ECM29 or siRNA-ECM29 cells, we did not observe any significant cell death/apoptosis resembling changes as measured by flow cytometry. Our new western blots for p21 and S139 phosphorylation levels of H2AX further support this.

What was the cut off used to define high versus low ECM29 mRNA expression (Fig. 5e-g)?

We used the default settings for the kmplot software. The cohorts are divided into two high/low according to the median expression of the measured mRNA.

What was the methodology used in the cell cycle analysis illustrated in Fig. 6c-e?

The method used was EtOH fixation followed by RNAse treatment and PI staining. We have now clarified this in our methods section.

3-
minor concerns that should be addressed
Line 31 - "is" instead of "are"

Thank you for pointing this out, the mistake has now been corrected.

Line 331 - Although the levels of ECM29 are about 50% reduced comparing to the WT cells (Western blot in Fig. 5c), it is not clear to the reader how did the authors conclude that the MCF7 CRISPR edited cells are heterozygous for ECM29 without a proper sequencing experiment to prove that.

The reviewer is absolutely correct in pointing this out. Unfortunately, when these experiments were done we did not have the resources to carry out the sequencing validation of the heterozygous state at the gene level, and the state is assumed based on protein expression levels shown in western blots. We have now corrected the text to be more precise about this. However, we would like to highlight that our conclusion that ECM29 is required for maintaining proliferation in breast cancer cells remains valid, as this experiment together with the siRNA based experiments show a substantial reduction in proliferation after targeting ECM29.

Line 1055 - "levels from Western blots in (g)" instead of "(e)"

Thank you for pointing this out, the mistake has now been corrected.

2nd Editorial Decision 19th Febuary 2018

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. It has now been seen by the three original referees, whose comments are enclosed below. As you will see, all referees find that their concerns have been sufficiently addressed and are now broadly in favour of publication, pending minor issues brought up by referee #3 are convincingly addressed.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending satisfactory revision of the remaining issues related to data representation.

REFEREE REPORTS

Referee #1:

I am very satisfied with how the authors addressed my comments and support publication.

Referee #2:

My previous review of this paper was highly positive (a relative rarity), and that opinion has not changed. I have evaluated the authors' response to other reviewers, and think they have responded appropriately to address their concerns. In my view the manuscript is suitable for publication.

Referee #3:

Miettinen et al. provide a revised manuscript in which the great majority of the comments and questions were addressed convincingly. However, the demonstration that the loss of ECM29 induces senescence remains insufficiently proven. Currently, there is no gold standard method for senescence detection and thus the molecular characterization of cellular senescence relies on the combined analysis of a panel of markers (see for example PMID: 27979832). In this revised version authors claim senescence induction based on the analysis of beta-galactosidase staining and a modest increase of phosphorylation of histone H2AX and p21 protein levels. Given the use of only three markers and the technical limitations of the experimental design (i.e., cell extracts harvested only 48h after ECM29 silencing, instead of the usual 5-7 days of senescence-inducing treatments), authors should tone down the claim of cellular senescence induction upon ECM29 loss. Instead, and in accordance with the provided data, ECM29 loss seems to induce a proliferation arrest phenotype associated with few senescence markers.
The beta-galactosidase staining could be illustrated with better quality pictures, e.g. WT control without the intense blue background. In addition, the legend of the western blot quantification in panels (f) and (g) should be more clear in explaining the standard deviation of the n=3. Are these 3 independent experiments? Three technical replicates? Three quantifications of the same band? Finally, it is not clear which loading control was used in this western blot experiment: beta-tubulin (indicated in the western blot) or alpha-tubulin (indicated in the quantification)?

2nd Revision - authors' response 2nd March 2018

We have performed all the requests of reviewer 3 and toned down the senescence induction throughout the abstract and the manuscript and added information about the number of independent experiments.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Mikael Bjorklund & Matthias Trost
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2017-98359

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	all experiments were performed at least in triplicate. Sample sizes were dependent on experiences with the technical and biological variability of the system analysed. For example, for experiments with smaller effects such as the proteasome activation assays, we performed more replicate assays.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	details about statistical tests have been added to each figure.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	bigger datasets such as the proteomics data were assessed for normal distribution using histograms.
Is there an estimate of variation within each group of data?	no. in most cases it was not possible to estimate variation for each group of data.
Is the variance similar between the groups that are being statistically compared?	it is assumed that variance between groups studied are similar.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies are given with theire catalog numbers and suppliers.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines were obtained from frozen stocks from the MRC PPU cell culture facility. These frozen stocks were generated from very early passages from cell lines acquired from ATCC. All cell lines were tested regularly for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Proteomics data has been deposited to PRIDE with the identifier PXD003704
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA